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Effects of pesticides on testes at ultrastructural and hormonal levels

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Abstract
Aim: Endocrine disruptors damage the functions of hormones in the body by imitating or blocking them. They and their metabolites change hormone levels and functions in the body. Pesticides constitute a significant group of endocrine disruptors. It is known that Profenofos, and 4-chloro-2-methylphenoxyacetic acid (MCPA) have negative effects on male genital system. However, studies about the effect on ultrastructural size are limited. Therefore, it is intended to compare the effect of MCPA and Profenofos on the ultrastructural level of the testes.
Material and Methods: There were three groups in the study (control, Profenofos, MCPA), each of which included ten fourteen-week-old male rats. Electron microscopy and biochemical investigation were performed on the excluded tissues of the testes.
Results: In histopathologic investigations, spermatogenesis was healthy in the control group. Structural degenerations were observed on spermatogenic cells and Sertoli cells in the profenofos group. The gaps among spermatogenetic cells, cellular degeneration (i.e. structural damage) in the MCPA group was more obvious than in the Profenofos group. Considering the biochemical results, a significant decrease in testosterone level was observed in the animals receiving both profonefos and MCPA.
Discussion: Profenofos and MCPA prevent the healthy continuation of spermatogenesis and therefore may cause infertility.

Keywords
Electron Microscopy, Endocrine Disrupter, Infertility, MCPA, Pesticides, Profenofos, Testes

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Introduction

A pesticide is any substance or mixture of substances used in animal husbandry, agriculture, and public health to repel, kill, or keep under control unwanted organisms (pests) such as insects, rodents, fungi, bacteria, and weeds. However, pesticides have toxic effects on the environment and beneficial living organisms. Pesticides also cause toxic effects on humans by leaving residues in soil, water, and food [1]. The toxic effects of pesticides on humans are separated into acute and chronic. The effects of acute poisoning range from nausea, vomiting, and dizziness to death. Chronic exposure gives rise to reproductive toxicity, endocrine system disorders [2, 3].

Profenofos and 4-chloro-2-methylphenoxyacetic acid (MCPA) are pesticides classified as class II (moderately) toxic substances by WHO [(avaible at : https://apps.who.int/iris/handle/10665/332193)]. Profenofos is an organophosphate class insecticide, and MCPA is a chlorophenoxy derivative herbicide [4]. Studies have proven that chlorophenoxy compounds are teratogenic and embryotoxic [5]. Bilateral and unilateral tubular degeneration was noticed in the testes of rats exposed to MCPA. Profenofos causes inhibition of platelet-activating factor acetylhydrolase (PAF-AH). PAF-AH is vital for brain development and testes. PAF-AH inhibition results in severe impairment of spermatogenesis. In studies conducted in rabbits, it was observed that Profenofos disrupts testicular structures, reduces body and testicular weight, creates gaps in seminiferous tubules, leads to destruction and hypertrophy of Leydig cells, and it was concluded that it is an endocrine disrupting chemical [6]. The use of Profenofos in combination with other pesticides increases its genotoxic and cytotoxic effects [7].

Material and Methods

This study was approved by Başkent University Animal Experiments Local Ethics Committee (Project Date: 25.08.2009; Project No: DA 09/30) and supported by Başkent University Research Fund. In the study, three groups of 14-week-old rats were formed with ten male rats in each group. On the first day, 0.5 ml of blood was taken from the tail veins of all animals to measure serum testosterone levels. Profenofos and MCPA doses were selected based on doses which are known to have cytotoxic effects [8, 9].

Group 1 (Control Group): In this group, only distilled water was administered via gavage. They were allowed free access to food and water for one month under normal conditions.

Group 2 (Profenofos Group): In this group, Profenofos (Pestanal, 45632-Fluka, Sweden) was administered to the subject by gavage at a dose of 17 mg/kg on certain days and hours twice a week for one month.

Group 3 (MCPA Group): In this group, MCPA (25190, Fluka, Sweden) was administered by gavage at a dose of 190 mg/kg every day on the specified day and time for one month.

All animals were sacrificed after anesthesia with 60 mg/kg ketamine and 10 mg/kg xylazine. Testicular tissue samples removed from the rats were cut into small pieces and placed in phosphate-buffered 2% glutaraldehyde fixative solution (pH 7.4) for electron microscopy examination. Blood samples taken from the animals on the first and last day were sent to the biochemistry laboratory to measure serum total and free testosterone levels. The tissue samples were fixed with glutaraldehyde and osmium tetroxide, embedded in Araldite, and blocked. From the blocks, 70-90 nm sections were taken on copper grids with a Leica ultramicrotome. The sections were stained with uranyl acetate and lead citrate and photographed under the LEO 906E EM.

All statistical analyses were performed with the IBM SPSS 21.0 package program. Before the analyses, the normality of the data was checked by the Shapiro-Wilk test and Q-Q graphs. Differences between initial and final measurements were analyzed by the Wilcoxon test, and the Kruskal-Wallis test analyzed differences between groups at each measurement time. Pairwise comparisons for parameters found to be significant after the Kruskal-Wallis test were analyzed by Dunn's test with Bonferroni correction. A significance level of 5% was taken for statistical tests.

Ethical Approval

Ethics Committee approval for the study was obtained.

Results

Electron Microscope Examination

TEM examination of tissue samples from the control group showed that spermatids, Sertoli cells and Leydig cells at different developmental stages exhibited normal structure, intercellular structures were preserved, and tissues were in normal course. The formation of heterochromatin-rich acrosomal vesicles was observed in round spermatids (Figure 1-A). In spermatids at an advanced stage of development, the nuclear chromatin is condensed, and the centrioles are located at the opposite pole of the developing acrosomal vesicle. In contrast, in spermatids approaching spermiogenesis, the midpiece is fully formed and the mitochondria are regularly arranged (Figure 1-B). Sertoli cells in the basal lamina of the seminiferous tubule and Leydig cells in the interstitial area were found to have normal structures (Figure 1-C). Sertoli cell located in the basal lamina was distinguished by its euchromatin-rich nucleus and prominent nucleolus, while mitochondria and a few electrons-dense granules were observed in the cytoplasm (Figure 1-D). Electron micrographs showed that Leydig cells in the interstitial connective tissue had a natural structure and were located close to capillaries (Figure 1-E). As a result of these findings, the spermatogenesis process was considered healthy.

TEM examination of tissue samples from the Profenofos group showed that some seminiferous tubules retained their normal structure, while others showed significant structural changes. It was observed that acrosome vesicle developed in some round spermatids in the tubules that preserved their integrity. The basal lamina lost its homogeneous structure. It was noted that in the tubules, where structural damage was observed, prominent spaces were formed between the spermatogenic cells, and the integrity of seminiferous epithelium could not be preserved (Figure 2.A). Significant swelling and cristaelysis were observed in the mitochondria of spermatids in the spermatogenesis stage, while the residual bodies in the cell cytoplasm were determined (Figure 2.B). While it was observed that the nucleus of the Sertoli cell lost its normal structure and was rich in heterochromatin in places, swollen cristaelysis mitochondria

and residual bodies were observed in the cytoplasm (Figure 2.C). It was observed that the basal lamina of the seminiferous tubule exhibited a wavy appearance in some areas; unlike the control group, there were occasional separations. While residual body formation, large lipid droplets, and widespread vacuolization were observed in the cells forming the walls of these tubules, it was noted that mitochondria had abnormal shapes such as bagels or notches with swelling and cristaelysis. The cytoplasm of Leydig cells in the intermediate connective tissue was less intensely stained (electron-permeable), with cristaelysis mitochondria and autophagic vacuoles (Figure 2.D). Moreover, with regard to the structural damage caused by Profenofos application in cells forming the spermatogenic series, it was observed that Profenofos application caused significant structural disruptions in Sertoli cells. Based on this thin section finding, it was determined that the blood-testes barrier was disrupted, and Profenofos negatively affected the spermatogenesis process.

In the testicular thin sections of the MCPA- administered experimental group, gaps were observed, as in the Profenofos group, between the cells forming the seminiferous tubule epithelium. In some of the spermatogenic cells, chromatin was found to be condensed under the nucleus membrane. The basal lamina of the seminiferous tubule showed a wavy appearance and regional thickening (Figure 3.A). In different field examinations, irregularities in the basal lamina were

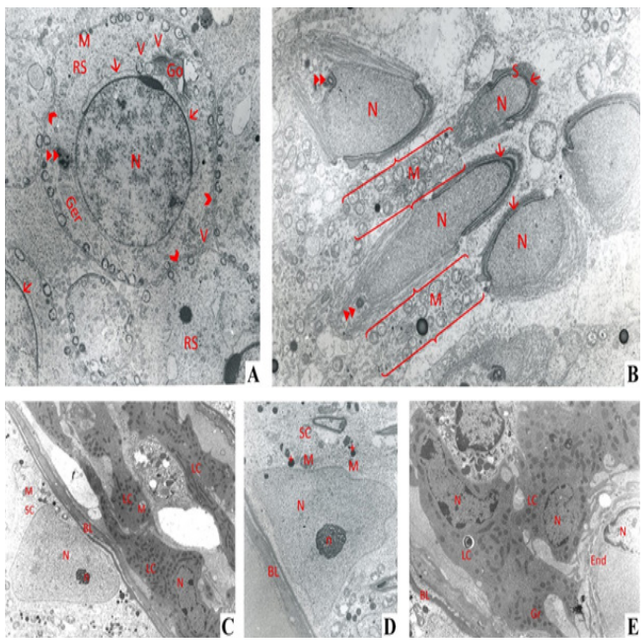

Figure 1. Electron micrographs of the control group.
A. In the round spermatid (RS) in the seminiferous wall, the nucleus (N) shows a developing acrosomal vesicle (→) at its anterior pole, Golgi complex (Go), vesicles (V) dispersed in the cytoplasm, mitochondria (M), autophagic vacuole (>>|), granulated endoplasmic reticulum (Ger) and corrugated connection (>) with other cells (X3597). B. Spermatids in advanced development (S) are characterized by condensed nucleus (N) chromatin, centrioles (>>) at the opposite pole of the acrosomal vesicle (→), and mitochondrion (M) regularly arranged in the mid-tail ([1]) of spermatids approaching spermiogenesis (X3597). C. Sertoli cell, located on the basal lamina (BL) of the seminiferous tubule, is distinguished by its euchromatin-rich nucleus (N), prominent nucleolus (n), and mitochondria (M) distributed in the cytoplasm. In the interstitial connective tissue, Leydig cells (LC) are observed with prominent nuclei (N) and nucleoli (n) (X2784). D. At large magnification, Sertoli cell (SC) on the basal lamina (BL) of the seminiferous tubule is visualized with euchromatin-rich nucleus (N), prominent nucleolus (N), mitochondria (M) and electron-dense granules (+) in the cytoplasm (X2784). E. High magnification shows the chromatin distribution of Leydig cell (LC) nucleus (N) and nucleoli (n), mitochondria (M), dense matrix granules (Gr) in the cytoplasm, and the normal-looking nucleus (N) of a capillary endothelial cell (End), located near the cells in interstitial connective tissue (X4646).

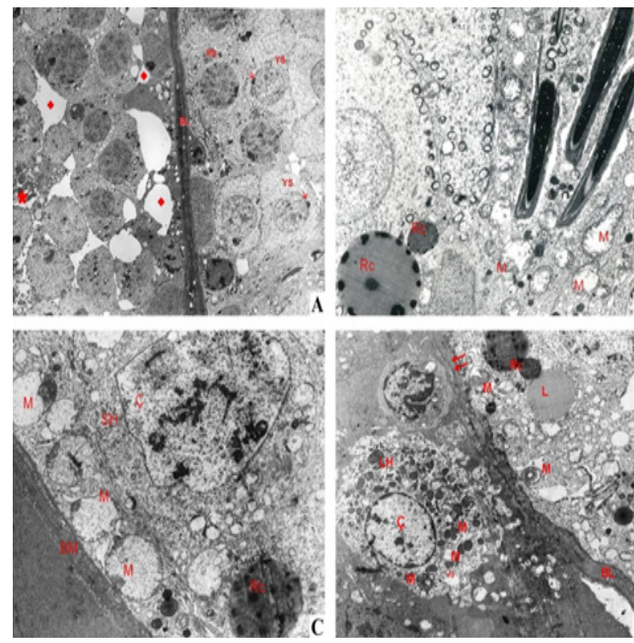

Figure 2. Profenofos group electron micrographs.
A. On the right side of the picture, the acrosomal vesicle (→) is seen in primary spermatocytes (PS) and round spermatids (RS) in the seminiferous tubule that maintains its integrity.
Electron-dense staining of the basal lamina (BL) forming the border of the two tubules can be distinguished. In the left half of the image, prominent gaps (◊) between the cells forming the seminiferous tubule wall and apoptotic formation (★) near the lumen are observed (X1293). B. Swollen cristaelysis mitochondrion (M) in the surrounding cells of spermatids at the spermatogenesis stage with the presence of residual bodies (Rb) in the cytoplasm (X1293).
C. High magnification showing abnormal Sertoli cell (SC) nucleus (N) on the basement membrane (BM), abnormally shaped, large and cristaelysis mitochondria (M) in the cytoplasm, and residual bodies (Rb) (X4646). D. The basal lamina (BL) of the seminiferous tubules was observed to be irregular (⇒) and separated in places. Residual body (Rb) formation, large lipid droplets (L), and extensive vacuolization (Va) were observed in the cells forming the wall of these tubules, while mitochondria (M) were abnormally shaped in the form of bagel-shaped or notched with swelling and cristaelysis. Less intensely stained Leydig cell (LC) in the intermediate connective tissue shows mitochondrion (M) with cristaelysis and autophagic vacuole (>>|) formation (X 2784).

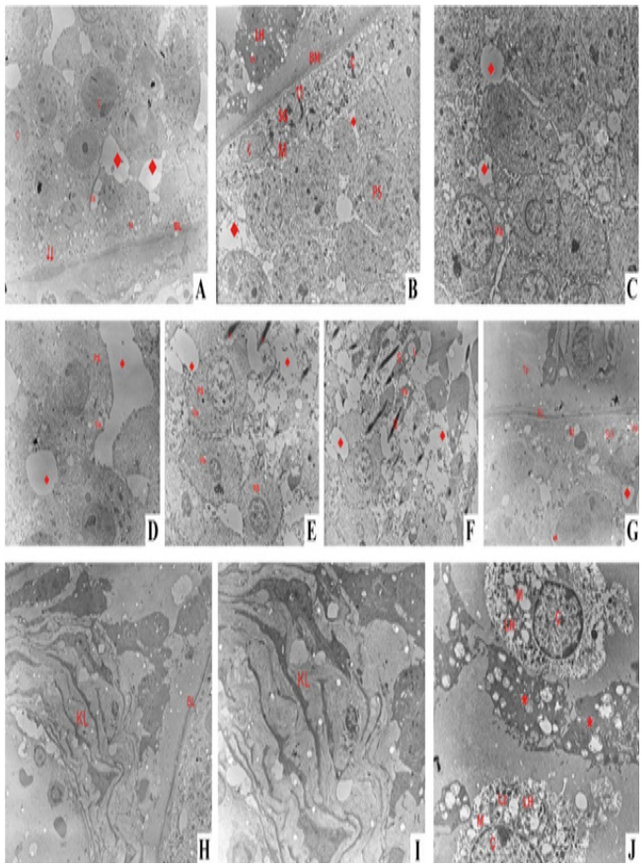

Figure 3. Electron micrographs of the MCPA group.
A. Irregular course (⇒) of the basal lamina (BL) of the seminiferous tubule in the testis, intercellular space (◊) in the tubule wall. In some spermatogenic cells at different developmental stages, chromatin is condensed under the nucleus membrane (N), vacuolization (Va) and cristaelysis mitochondria (M) are seen in the cytoplasm of some cells (X1293). B. In the seminiferous tubule, irregularities in the course of the basement membrane (BM) (⇒), gaps between cells (◊), cristaelysis in the mitochondria (M) of spermatogonium (SG) and spermatocytes (S), and vacuolization (Va) in Leydig cells (LC) with dark cytoplasm in the interstitial space (X1670). C. In tubules, where structural damage is evident, high magnification shows gaps between developing cells (◊) and vacuoles (Va) in their cytoplasm (X2156). D. High magnification showing vacuoles (Va) in the cytoplasm of primary spermatocytes (PS) and gaps (◊) between cells (X2784). E. In tubules with advanced structural damage, gaps (◊) between primary spermatocytes (PS) and late spermatids (S) can be recognized as having lost their connection with Sertoli cells (X2156). F. Some of the late spermatids (S) in the seminiferous tubule wall show lipid droplets (L), vacuoles (Va) accumulation and intercellular spaces (◊) in the cytoplasm (X1670). G. Spermatogonium (SG) located on the tunica propria (Tp) shows vacuolization (Va), mitochondrion (M) cristaelysis, intercellular spaces (◊), and dilatation (>>) of spermatocyte junctions (X2784). H. In the testis, tunica propria (Tp), collagen fiber (CF) increase and basal lamina (BL) are seen (X1670). I. Large magnification shows collagen fiber (CF) distribution (X2784). J. Leydig cells (LC) with clear cytoplasm, nucleus (N), mitochondria with cristae lysis (M), lysosomes (Lys) and apoptotic Leydig cells (★) in the interstitial space (X3597).

observed, although damage to the seminiferous tubule wall was less. Cristaelysis and intercellular dilatation were observed in the mitochondria of spermatogonium and spermatocytes. In this group, vacuolization was observed in the electron-dense cytoplasm of Leydig cells in the interstitial area (Figure 3.B). In tubules, where structural damage was evident, gaps between primary spermatocytes, vacuoles in their cytoplasm, and irregularities in cell borders were detected (Figure 3.C-D). Although late spermatids should normally be associated with Sertoli cells, they were observed to be discrete in some tubules (Figure 3.E). Accumulation of lipid droplets in the cytoplasm of some of the late spermatids was remarkable (Figure 3.F). In addition to degeneration in the spermatogonium, cristaelysis, swelling and vacuolization were observed in mitochondria. It was determined that intercellular spaces were enlarged, and spermatocyte junctions were expanded (Figure 3. G). In the examinations, increased collagen fibers were observed in the intermediate connective tissue (Figure 3.H-I). Apoptosis was detected in some of the MCPA- administered Leydig cells, apart from the Profenofos group. (Figure 3. J). Gaps between the spermatogenic cells, cellular degeneration, and structural damage in the cells were slightly more evident in the MCPA-administered group compared to the Profenofos group.

Biochemical Examination

There was no statistically significant difference between the initial (3.46 ± 1.73 pg/ml) and final (2.07 ± 2.80 pg/ml) measurements of free testosterone in the control group (p=.309), while there was no statistically significant difference between the Profenofos group (6. 33 ± 3.64 pg/ml vs. 1.85 ± 1.40 pg/ml, p=.011) and MCPA group (3.59 ± 1.98 pg/ml vs. 2.49 ± 2.16 pg/ml, p=.020). Similarly, there was no statistically significant difference between the initial measurements of total testosterone (223.91 ± 112.44 ng/ml) and the final measurements (109.11 ± 77.79 ng/ml) in the control group (p=.720). Total testosterone levels were significantly decreased in the Profenofos group (369.10 ± 234.62 ng/ml vs. 108.18 ± 53.88 ng/ml, p=.013) and MCPA group (222.85 ± 102.01 ng/ml vs. 201.62 ± 164.79 ng/ml, p=.045). On the other hand, while there was no statistically significant difference between the groups in the initial free testosterone values (p=.076), the final free testosterone level was significantly higher in the MCPA group than in the Profenofos and control groups (all p<.05), and the free testosterone level in the Profenofos group was significantly lower than in the control group (p<.05). However, for total testosterone, there was no statistically significant difference between the groups at both initial and final measurements (p=.265 and p=.141, respectively).

Discussion

The effects of organophosphates on spermatogenesis are well known. A study was conducted on rats to determine the effect of Profenofos on spermatogenesis at the ultrastructural level. Degenerative alterations were found in the testicular epithelium and Leydig cells [8]. There are studies reporting that Profenofos decreases sperm motility and count, and increases sperm anomalies [10, 11]. Histopathologic examination in these studies revealed an obstruction in the seminiferous tubules and decreased sperm count in the lumen.

In our study, degenerative changes in seminiferous tubule epithelium were found in electron microscopy examinations of the Profenofos group. In the seminiferous tubule cells, numerous large residual bodies, and lipid droplets, abnormally shaped, swollen and cristaelysis mitochondria with diffuse vacuolization were observed. In addition, mitochondrial degeneration and autophagic vacuoles were observed in Leydig cells in the interstitial tissue. Profenofos negatively affected spermatogenesis and large gaps were observed between the cells forming the spermatogenic series. Numerous residual bodies and advanced cristaelysis in mitochondria were also observed in Sertoli cells. Degenerative changes were also observed in the basal lamina of the seminiferous tubules, and in some tubules, the shape of the basal lamina was irregularized, and separations were observed. In Leydig cells in the intermediate connective tissue, increased matrix condensation in mitochondria, as well as swelling and vacuole formations, were observed. These degenerative structural phenomena, the biochemical and statistically significant decrease in testosterone, negatively affect spermatogenesis, prevent healthy sperm development, and may cause pesticide-mediated infertility. The decrease in serum cholinesterase was accompanied by a decrease in LH and FSH, while testosterone levels also decreased [11]. These data suggest that one reason for the significantly lower plasma testosterone level in our biochemical analysis is the decrease in gonadotropin-stimulating hormones in serum. Testosterone is secreted by interstitial Leydig cells in the testes under the influence of LH. Decreased testosterone levels may be due to direct damage to Leydig cells or decreased stimulation of these cells by LH.

Moustafa et al. treated Profenofos orally in male rats at a dose of 17.8 mg/kg twice a week for 65 days. They found that Profenofos caused significant edema between seminiferous tubules, structural damages in Leydig cells and vacuole formation in spermatogenic series cells in rat testes [8]. The effects of Profenofos in rabbit testes, such as decreased weight, shrinkage of seminiferous tubules, tumor-like structures, expansion in the interstitial space, reduction, or hypertrophy of Leydig cells were observed, and it was concluded that it is an endocrine disruptor due to the destruction of Leydig cells [6]. Our study has similarly determined gaps between the cells forming the seminiferous epithelium because of Profenofos and MCPA treatments.

In another similar study, the effects of Profenofos on male-specific cytochrome P450 (CYP) enzymes were investigated in Wistar rats. It has been reported that Profenofos, an organophosphate pesticide, is an endocrine disruptor of male specific CYP enzymes and causes a decrease in testosterone levels [12]. Our study measured free testosterone and total testosterone levels; It was determined that testosterone value decreased significantly in the Profenofos group ($p<0.05$).

Regarding histologic structure, the testes of rats administered with organophosphate pesticides had normal testicular structure, but congestion was found in seminiferous tubules [11]. In our study, it was observed that some seminiferous tubules in the Profenofos group preserved their natural structure, while in some seminiferous tubules, spermatogenic serial cells have severely degenerated. As a result of the experiment comparing the toxicology of MCPA and 4-chloro-2-carboxyphenoxyacid (CCPA) in rats, a decrease in the weight of epididymis and unilateral and bilateral tubular degeneration in testes were observed in rats given 190mg/kg MCPA [9].

In our study, degenerative changes were observed in the seminiferous tubule in the experimental group administered with MCPA in support of the studies mentioned above. These were cristaelysis in the mitochondria of spermatogonium and spermatocytes, along with irregularities in the seminiferous tubule basement membrane course. In these cells, there are gaps between spermatogonium and spermatocytes. Spermatids have lost their connection with Sertoli cells. Leydig cells in the interstitial area also showed abundant vacuolization. Some seminiferous tubules degenerated, while others retained their normal structure.

There are data in the literature that Profenofos causes sperm abnormalities; it has also been shown to cause a decrease in sperm motility and count. [10, 11]. Studies have shown that Profenofos covalently binds to tubulin proteins [13]. Disruption of microtubule formation because of the binding of Profenofos on α- and β-tubulin proteins may lead to disruptions or disorders in axoneme formation, which constitutes the most important part of sperm. This may explain the low motility of sperm with tail anomalies.

Conclusion

In our study we found that Profenofos and MCPA, whose acute effects we evaluated, caused irregularities in the basement membrane course of the seminiferous tubules and the formation of gaps between the seminiferous epithelial cells in the testicular tissue. In the Profenofos group, abnormally shaped mitochondria were observed in the testis cells. In the Profenofos group, Sertoli cell remnants were observed, whereas in the MCPA group, the germinal epithelium was found to have lost its connections with Sertoli cells. Cristaelysis was determined in the mitochondria of Leydig cells in the intermediate connective tissue. Statistical evaluation of free and total testosterone levels decreased significantly after administration. Although we think that this decrease is due to the endocrine-disrupting effects of MCPA and Profenofos, the mechanism is not fully understood and requires further investigation. The structural degeneration in the testicular appearance and the results of our biochemical data suggest that Profenofos and MCPA prevent the healthy functioning of the spermatogenesis process.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

The authors declare no conflict of interest.

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